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REVIEW

Plant viral disease management-from cross protection to CRISPR

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Abstract

In contrast to other plant pathogens, the control of viruses through chemical compounds is not feasible. Consequently, the management of plant viruses has predominantly relied on biotechnological approaches rather than those used for other pathogens. This paper presents a thorough review that takes into account an extensive literature analysis to offer a comprehensive understanding of biotechnological strategies aimed at developing stable engineered virus-resistant plants. Examples of these strategies were highlighted in crops, using as many cases as were available, where these strategies had been used, including cross--protection, pathogen-derived resistance (PDR), hairpin RNA, artificial small RNAs, and genome editing-based CRISPR/Cas systems. In evaluating the trends over time, we have critically assessed the advantages and disadvantages of each approach, identifying synthetic trans-acting microRNA (syn-tasiRNA) and CRISPR/Cas as the most effective and precise methods exhibiting minimal off-target effects on the plant genome. Furthermore, we have discussed the emerging innovations in non-transgenic resistance strategies such as the application of double-stranded (ds) RNA which hold promise for overcoming the significant challenges associated with the commercialization of engineered resistant plants.

Keywords: artificial small RNAs, hairpin RNA, RNA interference, topical dsRNA application, transgenic resistance

Introduction

At the turn of the 21th century about 1,000 plant virus species were reported (Bos 1999). However, since then this number has risen significantly, mostly due to applying metagenomics to discover novel plant viruses (Melcher *et al.* 2008)2008. In turn, the losses caused by viruses in crops are enormous. In terms of the significance of economic losses caused by plant viruses it was previously thought that they ranked third after that of fungal and bacterial diseases. However, given the fact that many plant viruses are asymptomatic and that increasingly new viruses are being discovered in crops and wild vegetation it is becoming evident that overall losses from viruses could take over that of other pathogens (Hilaire *et al.* 2022). Plant viruses annually account for $30 billion crop losses and 60% of plant diseases worldwide.

In contrast to the other pathogens, it is almost impossible to eradicate or control plant viruses by chemicals. It is true that compounds such as virazol (Ribavirin) were developed (formulated) long time ago (Agrios 2005) such compounds interfere with virus replication and at the same time may also affect replication of host nucleic acids. As in the case of *Potato virus X* (PVX), the inhibition occurs at an early stage of the virus replication (Lerch 1987). Ribavirin triphosphate is the inhibitory form of the compound and prevents viral RNA from capping. Capping is the joining of a guanosine nucleotide (NT) to the 5' end of RNA by a triphosphate bond. The cap endures viral RNA, however not all viruses possess the cap at their RNA's end and thus there is a limitation to its action in inhibiting viruses. Therefore, such compounds have

never been commercialized. This review provides information on all the virus-derived strategies with particular emphasis on crop case studies that have been carried out since the discovery of viruses and points at the evolving point(s) of these measures.

Cross protection

Cross protection, another name for mild strain protection, is a phenomenon where tolerance of a host plant to a virus is induced by prior inoculation with a mild strain of that virus or a closely related virus (Abdalla *et al.* 2018). Cross protection is the oldest measure for combatting plant viruses and was applied as early as 1929 (Fig. 1) (McKinney 1929) when the entity of a virus as a nucleoprotein particle had yet to be discovered. In the 1930s the building materials of viruses were explored (Pennazio *et al.* 2001). Cross protection resembles vaccination in humans and mammals though plants lack an adaptive immune system. Like other remedies the potential risks associated with its application were predictable at the time, such as reversion of a mild strain to a severe one, synergism with other virus(es), exhaustion of cellular metabolism as a result of pre-infection occupation of cellular sites by the mild strain, etc. However, because there were no other means to protect plants from viruses it was compelling enough to apply this measure.

As to the mechanisms of cross protection there are several hypotheses (Powell-Abel *et al.* 1989a, b), such as the speculation that a mild strain interferes with virus replication. Another hypothesis proposes that the mild strain depletes host cells of the materials such as nucleotides, amino acids and perhaps enzymes that are required for replication of the incoming virus. Yet another hypothesis proposes that the mild strain activity may end up in production of inhibitor compounds against the invading (challenge) viruses (Pennazio *et al.* 2001). Alternatively, the mild strain may preoccupy virus replication sites such as endoplasmic reticulum (ER) so that the challenge virus would be unable to settle in and start replication and infection (de Zoeten and Fulton 1975; Sherwood 1987; Urban *et al.* 1990). The earlier hypotheses were presented with only limited experimental support. A more acceptable hypothesis thinks that the coat protein (CP) of the mild strain prevents uncoating of the incoming severe strain (Powell-Abel *et al.* 1986). This is supported by an experiment where a mild strain of *Tobacco mosaic virus* (TMV) deficient in the CP did not produce protection (Sherwood 1987; Urban *et al.* 1990). Also, according to the same authors, the protection was broken when viral RNA was used as the inoculum. They suggest that uncoating is an important step in the protection. Moreover, another hypothesis speculates that the protection is RNA-mediated. In this scenario, the negative sense RNA strand of the mild strain which is produced during the virus replication anneals to the positive strand of the challenge virus thus sequestering it (Palukaitis and Zaitlin 1984). It should also be noted that the mechanism could vary depending on the plant-virus combination as reported by Gerber and Sarkar (1989).

A drawback for application of mild strain protection was observed in Australia. The mild strain protection scheme had been applied there in the 1960s against *Passionfruit woodiness virus* (PWV). Accordingly, a mild strain of PWV was deliberately inoculated on passionfruit seedlings and distributed to farmers in the northern part of New South Wales (NSW) and Queensland (QLD). By the 1980s, there were still such protected passionfruit vines growing in these regions. However, the occurrence of tip necrosis on the protected vines stimulated further research (Pares *et al.* 1985). In tip necrosis a synergy arises between the mild strain PWV and *Cucumber mosaic virus* (CMV). While such a dual infection ends up in the die back, infection with only PWV or CMV does not kill the vine. This led to applying coat protein-mediated resistance against these viruses (Sokhandan-Bashir *et al.* 1997, 2012).

Thus, the advent of recombinant DNA technology and the use of viruses as molecular vectors helped clarify the mechanism involved in cross-protection. If the coat protein were the determinate factor in the mild strain protein protection, the introduction of virus CP in the plant should bring about protection against the virus. This was the hypothesis for the production of stably transformed transgenic plants. For the first time, the CP gene from TMV was introduced into tobacco plants by *Agrobacterium tumefaciens* (*A. tumefaciens*). The resultant independent transgenic lines exhibited a wide spectrum of resistance from delay in symptom expression and lower quantity of virus to attenuation of symptoms and complete immunity (Powell-Abel *et al.,* 1986). This was the first indication of involvement of the CP in the resistance as manifested in the cross protection. As a further evidence, when the transgenic lines were challenged with naked TMV RNA, the protection became broken (Powell-Abel *et al.* 1989a, b). Later on (Grumet, 1995) it was reported that the mechanism of cross protection varied depending on the host–virus combination of the generation of transgenic TMV CP-expressing plants. This manifestation of the resistance was a turning point from mild strain protection to pathogen-derived resistance (PDR). In another case, delivery of mild strain TMV CP gene into tobacco plants even through PVX-based vector resulted in protection against a severe strain of the virus (Fig. 1) (Culver 1996).

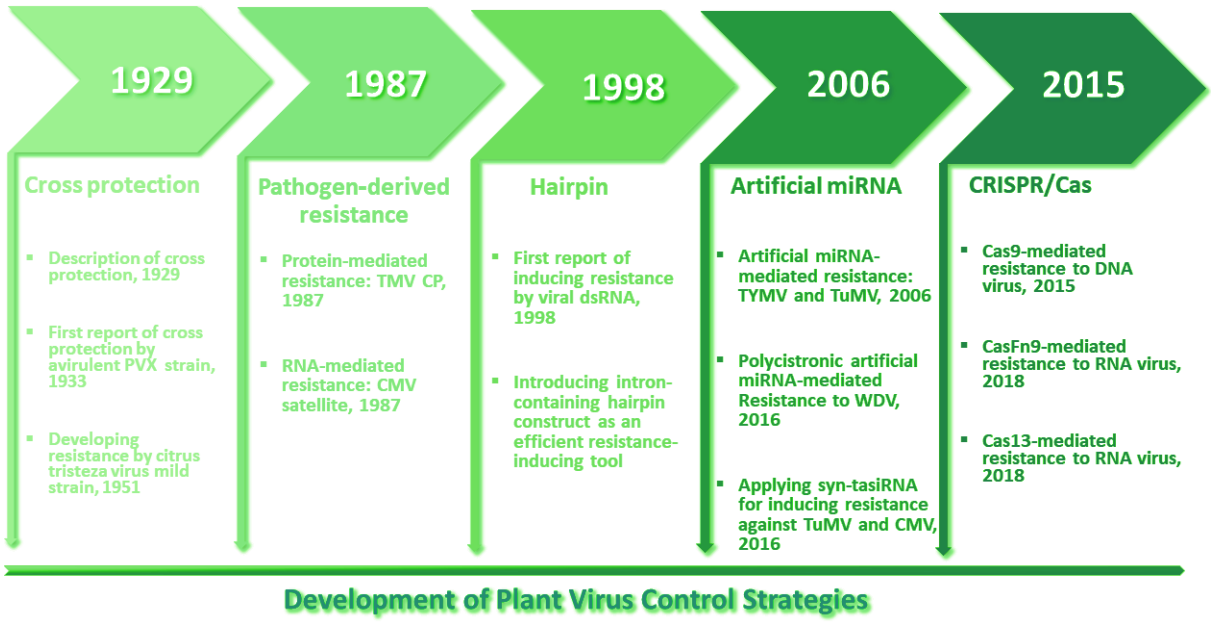

Fig. 1. Schematic diagram depicting the evolving timeline of resistance engineering strategies used against plant viral pathogens, from cross protection to the CRISPR/Cas system. Important periods for enhancing the proficiency in modern technologies of plant virus management are highlighted

Pathogen-derived resistance (PDR)

Subsequent to TMV CP- expressing transgenic plants which showed involvement of the CP in the protection (correlation between the level of the expressed CP and that of the resistance) (Powell-Abel *et al.* 1986) was the finding that even a truncated CP gene provides protection. Although this came from plants transgenic for N-terminal deficient CP of the potyvirus, *Tobacco etch virus* (TEV) this suggested that the PDR is not always based on CP but can also be mediated by another mechanism (in this case by CP RNA) in another host–virus combination (Lindbo and Dougherty 1992) By 1995 many transgenic virus- resistant plants were produced in different laboratories with various host--virus combinations and with different transgenes such as CP, movement protein (MP) and replicase protein (RP). The CP-mediated protection (CPMP) proved to provide wider protection, but the RP-mediated protection (RPMP) provided more efficient protection. It came to light that regardless of the mechanism, any segment of a virus genome if introduced into the host plant will make it resistant against the source virus and perhaps against closely related viruses (Grumet 1995). By then, not only the CP-mediated mechanism but also the RNA-mediated mechanism was demonstrated to be dependent on the plant- virus combination. However, more interesting were the cases where transgenic lines with more efficient resistance had lower amounts of transgenic RNA or did not have it at all. Speculation on the mechanism of resistance in such transgenic lines lacking transgenic RNA ended up in proposing an RNA-degradation model (Wassenegger and Pélissier 1998). Accordingly, when the transgene RNA level reaches a certain point an RNA- degrading mechanism starts to act with the involvement of an aberrant RNA. Further investigations into the mechanism of the RNA–degradation ended up in discovering the RNA silencing phenomenon (Ruiz *et al.* 1998). At about the same time, this phenomenon was also observed in transgenic petunia plants wherein putting multiple copies of the flower color-coding gene in the plant to change the color from pink to violet surprisingly resulted in flowers with no coloring (bleached) (Napoli *et al.* 1990).

RNA silencing

This phenomenon, depending on the organism, is known by different names including transcription gene silencing (TGS) in plants and animals, post transcription gene silencing (PTGs) in plants, quelling in the fungi and RNA interference in animals (Romano and Macino 1992; Fire *et al.* 1998; Waterhouse *et al.* 1998; Wei Ding 2000; Weinberg and Morris 2016). This phenomenon is triggered by small double--stranded RNAs (dsRNAs) about 22-30 bp in size with

2 NT 3′ overhangs. The origin of these small dsRNAs could be dsRNA and, in this case, they are called siRNA (short interfering RNA) which could be that of RNA viruses either ssRNA or dsRNA plant viruses. This is because ssRNA viruses make dsRNA as a replicative form during replication. Viruses can be the origin or target of the silencing. For example, in transgenic plants, the transgene RNA can anneal to invading virus RNA and form dsRNA which will trigger silencing. However, the presence of a virus in a plant can also be a source of silencing. For example, the viral RNA can form dsRNA after annealing to an mRNA which contains sequences similar to that of the viral RNA. This will lead to silencing of the endogenous gene. Alternatively, intra-molecular RNA base pairing in viral RNA can induce a dicer to cut the viral RNA at the paired region and produce microRNAs (miRNAs) as the source of the silencing phenomenon. Or, the replicating virus may silence other viruses which have NT similarities with it. After the discovery of RNA silencing (RNAi) it has many applications such as determining phenotype of a gene. Here, its applications in establishing protection to plant viruses have been focused on.

Alternatively, single RNAs native to a host cell can form small dsRNA as a result of intra molecular base pairing. Then the paired segment of the ssRNA molecule can act as a trigger. In this case, these small dsRNA molecules are named as miRNA (microRNA). In some organisms such as the fungus *Neurospora* sp, the plant *Arabidopsis thaliana* (*A. thaliana*) and the nematode *Caenorhabditis elegans* (*C. elegans),* because of owning RNA-dependent RNA polymerase (RdRp), can produce small dsRNAs as well (Wei Ding 2000). These small dsRNA, no matter what their sources are, will induce dicer which is type III-like endonuclease that act on dsRNA and cleave into microRNA (miRNA). Then, the short dsRNA is recruited to RNA-induced silencing complex (RISC) (Fig. 2). At this stage one strand of the short RNA is disassociated (passenger strand) leaving the other strand in RISC. Now the silencing complex will target any nucleic acid (DNA or RNA) which has similarity to the short RNA. It will degrade mRNA into smaller RNA fragments, prevent translation of miRNA which has similarity to the short RNA or even affect the corresponding DNA in the chromosomes which again have similarity to short RNA.

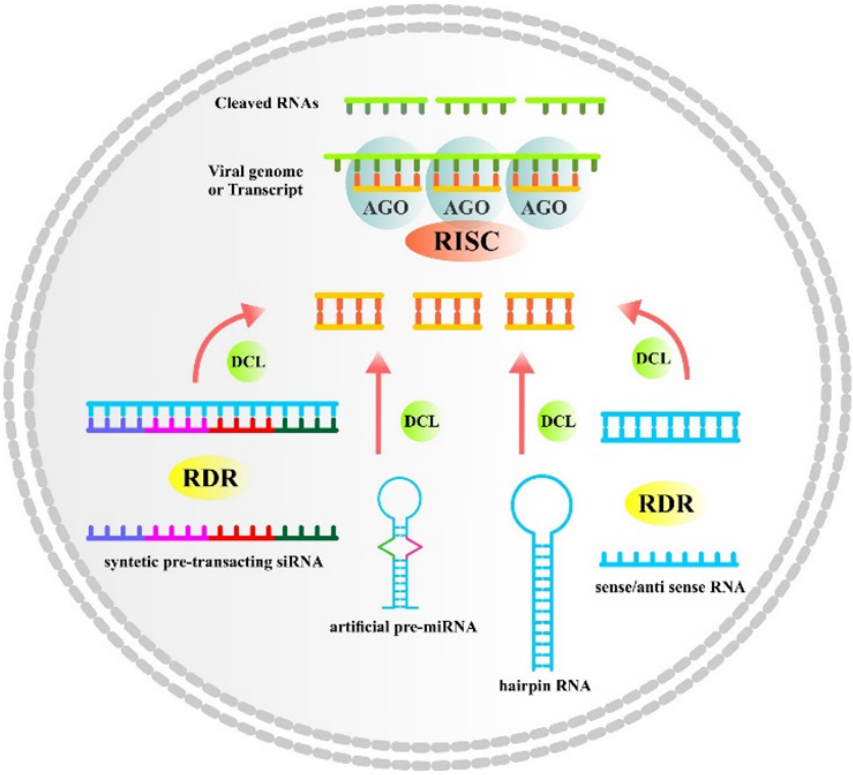

Fig. 2. Schematic illustration of RNA silencing-based approaches used to engineer plants for virus resistance. The mechanism involves triggering RNA interference machinery by introducing virus-derived artificial small RNAs, such as artificial pre-transcribing siRNA, artificial pre-miRNA precursors, hairpin or sense/antisense RNA. In the case of pre-transcribing and sense/antisense the second strand is synthesized by RDR (RNA-dependent RNA polymerase) which naturally exists in some organisms such as Arabidopsis thaliana. These dsRNAs are cleaved by DCL (dicer-like enzyme) to produce double strand short interfering RNA (siRNA)s or microRNA (miRNA)s which are loaded by Argonaute protein (AGO) to create RNA-induced silencing complex (RISC) in which one strand of the small dsRNAs is detached (passenger strand) but the remaining guide strand directs RISC to the target viral RNA or any other RNA that has similarity with the guide RNA and degrades it

Such stretches of DNA will become methylated, sequestrated and will be transcribed. The other interesting point is that the smaller fragments resulting from degradation of mRNA form short dsRNA and amplify the silencing (Li and Ding 2006).

Hairpin technology

Hairpins are pieces of RNA molecules, such as that of a viral RNA which act as sources of RNA silencing. Hairpins are designed according to virus RNA sequences. A hairpin has a structure similar to a stem loop structure (Fig. 2). In an RNA molecule, if base pairing occurs between two interspersed stretches of nucleotides (palindromes) then a hairpin RNA (hpRNA) can form. Such a stem loop structure will induce dicer to degrade it and form miRNAs that target RNA molecules containing a homologous sequence to that of the miRNA (Fig. 2).

HpRNA has become as a technology that helps induce RNA silencing more efficiently. This technology provides a stable dsRNA that is ready for primary siRNA processing, which in turn produces a large amount of secondary active siRNAs. This intensifies the RNAi transitivity, leading to systemic silencing once a viral infection is established (Waterhouse *et al.* 1998; Duan *et al.* 2012; Lindbo 2012; Zhao *et al.* 2020). It has been employed to compensate for the low performance of the sense and antisense constructs as they confer unstable and delayed resistance (Fig. 1) (Duan *et al.* 2012; Duan *et al.* 2008; Lindbo 2012; Zhao *et al.* 2020).

Delivery of hpRNA into a plant

A hpRNA transcribes from a gene expression cassette consisting of a promoter followed by an inverted repeat sequence of the intended target separated by a non-related sequence (spacer) placed in the center of the cassette and ended by a terminator. So, hpRNA appears as two complementary arms and a terminal loop, which is capable of underlying RNAi pathways. Studies on the efficacy of different hairpin-based RNAi triggering structures have shown that constructs containing intron spacers frequently achieved perfect silencing. This is because the intron splicing process promotes the formation of dsRNA, leading to a stabilizing effect on the hybridization reaction (Fig. 1). The nucleotide structure, which consists of a hairpin shape and is formed by the splicing of introns, is known as intron-spliced hpRNA (ihpRNA) (Smith *et al.* 2000; Wesley *et al.* 2001).

Applications of hpRNA

The hpRNA technology has rapidly evolved for silencing desired RNA in crop improvement programs, with an emphasis on resistance to the viral pathogens, which has gained popularity (Fusaro *et al.* 2006; Lindbo 2012; Zhao *et al.* 2020). In 2007, an experiment was conducted to develop a source of viral resistance in woody perennial plants. For this purpose, various *Plum pox virus* (PPV) coat protein (CP) ihpRNA constructs which had previously been successful in herbaceous model plants were evaluated for their ability to activate the functional anti-PPV RNAi pathway in plum. Additionally, tissue-specific promoters were occasionally manipulated to express in *Rosaceae* plants. A high level of resistance was observed with an ihpRNA construct containing full length PPV CP coding region under both the 35S constitutive promoter and peach *chlorophyll a/b-binding (*Cab*) protein gene* promoter. These results highlight the potential of the ihpRNA-expressing plum cultivar for commercial uses (Hily *et al.* 2007). RNAi induction was also high for an ihpRNA construct targeting p23 gene and 3' UTR of *Citrus tristeza virus* (CTV) compared to sense and anti-sense based transgenes in *Citrus aurantifolia* (*C. aurantifolia*) (Lopez *et al.* 2010). However, hairpin-mediated resistance was only observed in approximately one-third of the transgenic lines, with a wide spectrum of resistance ranging from 9 to 56%. This may be due to the fact that the hairpin-induced RNAi pathway corresponds to the viral defense pathway of plants and can be repressed by viral protein suppressors (Fusaro *et al.* 2006). However, *Banana bunchy top virus* (BBV) replication initiator protein (Rep) protein successfully fights the viral infection in banana plants transformed with ihpRNA constructs (Shekhawat *et al.* 2012).

Herbaceous crops expressing antiviral hairpins have shown resistance against viruses. The first transgenic tomato cultivar was generated to prevent a geminivirus, *Tomato leaf curl virus* (TYLCV) (Fuentes *et al.* 2016). The transformed tomato plants had an ihpRNA sequence that included almost 800 nucleotides from the 3' end of the TYLCV C1 gene. Accordingly, these tomato lines were fully resistant to TYLCV even when exposed to the virus in the field under conditions mimicking whitefly transmission[1]. In another case, transgenic cotton plants gained resistance to *Cotton leaf curl Multan virus* (CLCuMV) with the help of C4 gene-derived ihp RNA (Baig *et al.* 2021). The transgenic cultivars exhibited a common resistance trait against *Cotton leaf curl virus* (CLCuV) which had a significant effect on reducing the viral titer and delaying the

[1] Whiteflies are important hemipteran insect vectors for transmission of destructive plant viruses including *Geminiviridae* family members

appearance of symptoms. Hence, mixed infections are common features of viral infections in crops (Sokhandan-Bashir *et al.* 2012; Wieczorek and Obrępalska-Stęplowska 2013; Syller 2020). To achieve broad-spectrum resistance, ihp constructs have been improved to combat multiple viruses in a shared host. For instance, a single construct was created to transcribe three inverted hairpin structures. These structures individually contained conserved motifs of the *Alfalfa mosaic virus* (AMV), *Bean pod mottle virus* (BPMV), and *Soybean mosaic virus* (SMV) replicase coding region. The resulting siRNAs provided complete protection in bean plants against their respective viruses. This led to the generation of a line with a broad-spectrum viral protection trait (Zhang *et al.* 2011). Also, researchers have identified an effective target gene, Pns9, for combating *Reoviridae* family species that infect rice plants. They found that transgenic rice expressing hpRNAs of *Rice gall dwarf virus* (RGDV) Pns9 exhibited a high level of resistance with a significant accumulation of specific siRNA. This resistance was also stably inherited in progenies, making it a promising strategy for fighting RGDV infections in rice crops (Shimizu *et al.* 2012). Several promising results have been observed with hpRNA-mediated protection, but with different levels of protection in the model plants. This included partial resistance, breaking resistance, delayed infection, and low levels of resistance (Dalakouras *et al.* 2011; Duan *et al.* 2012, 2008; Kalantidis *et al.* 2002). The nonspecific effect of RNA silencing has been identified as the most critical drawback of RNAi which raised the most concern in RNAi research and the commercialization of genetically modified organism (GMO) products (Senthil-Kumar and Mysore 2011; Pooggin 2017).

RNAi transgenic plants are able to counter defense against viruses through Argonaute proteins (AGOs) containing virus-derived small interfering RNAs (vsiRNAs) mediating target specific RNA dicing (PTGS). To achieve a transgenic plant with stable PTGS, four steps must be followed: (1) accurately design the RNAi construct to carry an appropriate trigger sequence and produce optimized dsRNA, (2) ensure the competency for cleavage by Dicer to produce siRNA, (3) incorporate siRNA into the RNA-induced silencing complex (RISC) and (4) recognize and cleave the target. RNAi-expressing plants have the off-target effect that has two negative aspects. Firstly, it can induce a change in the crop phenotype or metabolites. Secondly, it can have an effect on other crops through vertical gene transfer. In all the steps mentioned, there is the possibility of off-target effects that, except for the first step, the rest are beyond the control of researchers (Senthil-Kumar and Mysore 2011; Akbarimotlagh *et al.* 2023). The first step involves selecting a suitable vector with an appropriate promoter that produces an optimal amount of siRNAs to avoid disordering the plant's RNAi regulation system (Wesley *et al.* 2001; Senthil-Kumar and Mysore 2011). The properties of the trigger sequence used to produce initial dsRNA are considered to be the most crucial aspect. Specifically, a high degree of similarity between the selected trigger RNA sequence and the intended target RNA is critically required for efficient and highly specific interference of the target sequence (Senthil-Kumar and Mysore 2011; Akbarimotlagh *et al.* 2023). It is noteworthy that selecting a sequence at the 5' untranslated region (5'UTR) of the target RNA sequence can significantly reduce non-specific effects. While a longer trigger sequence, which produces initial dsRNA, is directly correlated with RNA interference (RNAi) efficiency, it also has the potential to increase the likelihood of off-target effects (Tang *et al.* 2006; Filichkin *et al.* 2007; Higuchi *et al.* 2009; Senthil--Kumar and Mysore 2011). The quantity of synthesized dsRNA must remain below the optimal threshold to minimize the risk of off-target reactivity. The quantity of dsRNA generated is contingent upon the formation of secondary dsRNA *via* amplification mediated by RNA-dependent RNA polymerase (RDR). Furthermore, the amount of dsRNA that arises is affected by the site of construct integration within the plant genome, which is determined by the transformation technique employed. To a greater extent, the positional effect of the integrated transgene within the host genome can lead to silencing or the production of aberrant RNAs as well as an increased generation of dsRNA. Furthermore, it is advisable to maintain a single copy of the transgene as multiple copies may enhance the silencing potential of the non target active locus (Voinnet *et al.* 1999; Sijen *et al.* 2001; Himber *et al.* 2003; van Houdt *et al.* 2003; Bleys *et al.* 2006; Filichkin *et al.* 2007).

Why is hairpin technology overtaken by miRNA?

Hairpin technology was followed by microRNA (miRNA) technology basically due to the problems associated with a longer size of the hairpin than that of miRNA. The use of binary vectors in hairpin technology raises biosafety concerns due to the possible insertion of non-T-DNA regions of the bacterial plasmid DNA backbone, as noted by Oltmanns *et al.* (2010). Another issue is the use of a strong promoter, which may saturate RNAi pathways with a high number of produced dsRNA and alter the plant expression pattern. Hairpin technology has a trigger sequence length of 250–350 bp, which is considered as being very long and increases the likelihood of unspecific effects. When the size is bigger the number of short RNAs resulting from hairpin will be numerous, and some of

such siRNAs may potentially silence off target regions in the host genome. Also, under mixed infection conditions, there is a risk of a recombinant virus emerging that carries a genomic segment from non-target viruses or that suppresses transgenic-induced silencing by non-target or aggressive target virus strains in a synergistic interaction (Pooggin 2013; Fuentes *et al.* 2016; Syller 2020; Khoshnami *et al.* 2023). The other issue is in the synthesis of the hairpin and the cost involved. The shorter the RNA the less is the cost. There are many examples where application of miRNA has resulted in successful remediation of virus infection in plants and there are quite a few cases where such a strategy has gained commercial momentum.

Progress has been made in developing RNAi plants against viral infection using artificial miRNA for the mentioned reasons (Senthil-Kumar and Mysore 2011; Duan *et al.* 2012; Carbonell *et al.* 2014; Pooggin 2017; Carbonell *et al*. 2019a; Cisneros and Carbonell 2020). The advantages of artificial miRNA strategy will be discussed in the next section.

Artificial small RNAs (art-sRNAs)

Artificial small RNAs (Art-sRNAs), such as artificial miRNAs (amiRNAs) and synthetic trans-acting siRNAs (syn-tasiRNAs), are based on endogenous miRNAs and trans-acting small interfering RNAs, respectively. These small RNAs play a crucial role in regulating gene expression in eukaryotic organisms through RNA interference pathways (Chen 2009; Carbonell *et al.* 2014; Zhang 2014; Carbonell and Daròs 2017; Mengistu and Tenkegna 2021). Art-sRNA emerged as a tool because it offers several advantages over hairpin technology due to its specific and efficient performance which makes it superior to hairpin technology in the following ways. Firstly, the constructs prepared for maturation are designed to follow the early steps of the endogenous miRNA generation pathway, which ensures that the number of releasing siRNA is under the control of the endogenous miRNA generation machine (Carbonell *et al.* 2014; Zhang 2014; Carbonell and Daròs 2017; Carbonell *et al*. 2019a; Cisneros and Carbonell 2020; Kotowska-Zimmer *et al.* 2021; Das *et al.* 2023). Although this approach may make miRNA pathway saturated it is considered to be safer and more efficient than hpRNA. Secondly, computational automate assays guarantee both specificity and efficiency by designing a precursor with the following properties: the minimum potential of plant transcriptome targeting, a high degree of complementarity with desired RNA and no mismatch in seed region eight nucleotides and optimum entropy of the amiRNA/target RNA interaction (Carbonell *et al.* 2014; Fahlgren *et al.* 2016; Cisneros and Carbonell 2020).

Artificial miRNAs (amiRNAs)

MiRNA canonical biogenesis pathway starts with a transcription from DNA intron or intergenic region and generates primary miRNA (pri-miRNA) (Fig. 2). Then, after processing by nuclear RNase III enzyme DROSHA, the generated pre-miRNA shuttles from the nucleus to the cytoplasm, and is sequentially processed into mature miRNA duplex through RNase III endonuclease Dicer (Dicer-like 1)-mediated removal of the terminal loop (O'Brien *et al.* 2018; Kotowska-Zimmer *et al.* 2021). An AGO1 protein, the first component of minimal miRNA-induced silencing complex (miRISC) (Argonaute miRISC Component 1), detects the released miRNA duplex and the guide strand directs RNAi-mediated degrading or repression of recognized highly complementary target sequence (Fig. 2) (O'Brien *et al.* 2018; Kotowska-Zimmer *et al.* 2021). As pioneers in plant antiviral research, Niu *et al*. (2006) discovered that using Arabidopsis endogenous miRNA precursor pre-miRNA (mir 159) with a complementary sequence to *Potiviridae* family members led to a successful resistance phenotype without affecting miRNA biogenesis. Plants that received amiR-P69^{159} and amiR-HC-Pro 159 constructs were able to resist *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV), respectively, even at low-temperature conditions (15°C) (Niu *et al.* 2006). Since then, many researchers have attempted to optimize efficient and specific plant viral control by taking advantage of this approach.

Applications of amiRNAs

To extend successful examples in crop plants, we can start with transgenic tomato plants that express anti-CMV amiRNAs. Zhang *et al.* (2011) have developed transgenic tomato lines that block the amplification of CMV, both alone and mixed with other RNA viruses. This was achieved by expressing amiR-2a/b and amiR-3U, respectively, which target the overlap region of 2a and 2b protein genes and the untranslated 3' region of CMV RNA2. Additionally, they confirmed that the amiRNAs have a cell-autonomous function and do not move systemically (Zhang *et al.* 2011). Tomato plants have been successfully engineered to express amiRNA which can silence overlapping regions of AV1 (coat protein) and AV2 (silencing suppressor protein) of *Tomato leaf curl New Delhi virus* (ToLCNDV). These plants have shown high resistance to the virus which has been inherited by the T2 progency. The amiRNA's intelligent target site selection, which is highly conserved among *Geminivirdae* members, was the key to their effectiveness beyond their primary functions (van Vu *et al.* 2013). AmiRNA also showed significant

effectiveness in combating economically important viruses in monocotyledon crops. A polycistronic amiRNA made up of three potent individual Rep/RepA motifs from *Wheat dwarf virus* (WDV) conferred strong and heritable resistance in *Hordeum vulgare* (*H. Vulgare*). Transgenic barley exhibited durable and temperature-independent antiviral properties in the field with moderately cold temperature, a condition that favors the insect vector (Kis *et al.* 2016). Consistent with the previous case, a rice polycistronic amiRNA with five anti-*Wheat streak mosaic virus* (WSMV) arms showed durable antiviral effects in wheat plants. Transgenic wheat progenies displayed full resistance as well as intermediate resistance and complete susceptibility to WSMV. T2 progenies of the transgenic events confirmed the elimination of the virus without the presence of the marker gene, ruling out the escape of viruses[2]. Therefore, it can be inferred that utilizing a multi-targeting polycistronic amiRNA-based antiviral approach would enhance the sanitation method by establishing immune defenses even without the genetic markers (Fahim *et al.* 2012). Engineered rice miRNA precursors were used to create transgene lines with varied silencing abilities against *Rice stripe virus* (RSV) and *Rice black-streaked dwarf virus* (RBSDV). The constructs were validated and tested for stable expression in rice plants to degrade the target viruses' CP motifs. Transgenic plants containing pre-amiRNA 3' UTR of the CP gene showed better prevention of multi-infection, indicating optimized miRNA-based resistance against simultaneous virus infections (Sun *et al.* 2016). Alternatively, targeting the MP gene proved to be more successful in enhancing rice resistance to RSV than using amiRNA technology. Careful selection of target sequences and modification of the MP gene without markers resulted in improved resistance. These transgenic rice plants meet biosafety regulations and have reduced off-target effects, making them ideal for commercial use (Zhou *et al.* 2022).

Transgenic strategies have gained significance in creating disease-resistant perennial plants as traditional breeding methods face limitations. Few studies focus on high-performing amiRNA constructs for antiviral resistance in perennial fruit plants due to slow growth and complex genomes. Endogenous miRNA information from next generation sequencing (NGS) is scarce, and the elaborate process of stable gene transfer in woody plants adds to these challenges (Flachowsky *et al.* 2009).

A study has shown that using a combination of amiRNA and siRNA can help protect plum trees from the PPV. It has been found that using amiRNA alone was not efficient enough to prevent PPV infection in *Prunus domestica* (*P. domestica*). However, when siRNAs and amiRNAs were applied together, it resulted in efficient resistance to the virus in the natural perennial host. Effective protection against the PPV underscores the significance of functional collaboration between these two RNA molecules (Ravelonandro *et al.* 2019). Regarding the development of miRNA-based resistant sources to *Grapevine fanleaf virus* (GFLV), engineered Arabidopsis mir319[3]-based construct was found functional in grapevine embryos although it has not been assessed by virus challenging (Jelly *et al.* 2012).

Synthetic trans-acting siRNAs (syn-tasiRNAs)

Single-site targeting of viral genomes by amiRNAs can lead to the emergence of new viral escapes. To overcome this, a new antiviral tool called syn-tasiRNAs has been developed on the basis of the multiplexing properties of transacting RNAs tasiRNAs for multi-targeting of the gene of interest or gene's transcript (Carbonell *et al.* 2014; Zhang 2014; Carbonell and Daròs 2017; Carbonell *et al.* 2019a; Cisneros *et al.* 2023).

Apart from the contribution of AGO1, the biogenesis of transacting RNAs (tasiRNA) differs from that of miRNA. Upon being processed by the miRNA/AGO complex, the primary transcript of tasiRNA or TAS precursor is amplified by RDR6 to create a template for synthesizing dsRNA. Following this, DCL4 cleaves the resulting dsRNA at the miRNA-cleaved site, generating 21-nucleotide phased duplexes. AGO1 loads the syn-tasiRNA guide strand selectively, which starts with a 'U' at its 5' end, to target one or multiple sites in at least one specific viral RNA. Typically, the non-preferred strand (passenger strand) of AGO1 is degraded (Zhang 2014; Cisneros and Carbonell 2020).

Applications of syn-tasiRNAs

For the first time in plant research, transgenic *A. thaliana* was generated that targeted two phylogenetically distinct RNA viruses in parallel to increase the resistance level. Accordingly, a modified Tas3 conserved gene expressing six ata-siRNAs against CMV and TuMV, preserving an essential 5' cleaving site, is capable of clearing both viruses in the *A. thaliana*. To expand the findings of Chen *et al.* (2016), a comparative analysis was performed between an antiviral tool that employs synthetic tasiRNAs and amiRNA for managing two isolates of the *Tomato spotted wilt virus* (TSWV). Interestingly, data derived from transient expression in *N. benthamina* showed that the

[2] Virus variants that have overcome the RNAi-resistance through mutation in the silencing target sites

[3] MiR319 is a type of microRNA found in plants that plays a role in regulating various processes related to plant development

performance of the syn-tasiRNAs multi-targeting construct was equivalent to that of the most efficient amiRNA construct, successfully blocking both TSWV isolates (Carbonell *et al*. 2019b). Results from another comparative analysis showed that the syn-tasiRNAs approach was more advantageous than amiRNAs in providing robust and long-lasting protection against TSWV by means of stable expression in crop plants. In contrast to the plants expressing anti-TSWV syn-tasiRNAs, the transgenic tomato plants with an anti--TSWV amiRNA construct allowed the viral progeny to escape resistance by exploiting mismatches in critical target sites (Carbonell *et al.* 2019a). As mentioned earlier, viruses can evolve more easily to become resistant to plant defenses that use a single-target strategy than to those that use multi-targeting strategies. In a study, two RNA silencing strategies using amiRNAs were optimized to achieve more precise silencing (Cisneros and Carbonell 2020). They have created a new plant binary vector by relocating syn-tasiRNAs closer to the trigger miRNA target site, leading to enhanced accumulation of released siRNAs and stronger gene silencing in *A. thaliana*. A single mismatch at the 3' end of artificial sRNA was found to be permissible for efficient silencing maintenance. The accuracy of these strategies was also applied against a plant virus, TSWV (López-Dolz *et al.* 2020)2020. The potential of artificial sRNAs to control viroids has also been examined. Researchers tested several amiRNAs and syn-tasiRNAs constructs in *N. benthamiana* to silence motifs in the conserved, variable and stem regions, as well as the internal loop of the *Pospiviroid potato spindle tuber viroid* (PSTVd). The study found that both types of constructs effectively suppressed the viroid, promising potential tools for managing PSTVd (Carbonell and Daròs 2017). Due to just recent emergence of these tools in plant virus and viroid control, currently there are yet no examples of commercial applications of syn-tasiRNA for controlling plant viruses.

Designing Art-sRNAs

The web-based P-SAMS and WMD3 platforms facilitate the design of efficient and specific amiRNAs and syn-tasiRNAs by streamlining the process and reducing costs. P-SAMS automatically generates art-sRNAs by computing specific complementation with desired sequences. It ensures no mismatches in the 5' seed region that contains a U nucleotide, and also includes a C nucleotide in the 19th position to result in a star sequence with a 5' G which helps to avoid AGO1 loading of the sequence. The MiRNA cleavage site between nucleotides 2-13 in the 5' region of art-sRNAs allows for preferential and specific loading into Ago1. The hybridization energy of the art-sRNA-target interaction is computed by WMD3 and optimized to be 35–40 kcal · mol^{-1} (Schwab *et al*. 2006; Fahlgren *et al.* 2016).

In 2014, Carbonel and colleagues created highly efficient art-sRNAs by expressing them through specific vectors that allowed for one-step cloning. The vectors contain a modified version of either MIR390a or trans-acting precursor cassette which is designed to embed the art-sRNAs oligonucleotide (designed at http://p-sams.carringtonlab.org) with flanked *Bsa*I restriction enzyme overhangs on both sides located in the *ccdB* region (Carbonell *et al.* 2014)2014. More recently, in an attempt to optimize amiRNA-based biotechnological tools, researchers have discovered an miRNA precursor only 89 NT in length which can induce systemic silencing in *N. benthamiana* genes through non-transgenic methods. This minimal successful construct could be a breakthrough in modern RNAi--based antiviral tools because it simplifies the generation of the construct and enables *in vitro* production for the aforementioned non-genetically modified plant protocol (Cisneros *et al.* 2023).

Apart from designing the construct, selecting a proper target viral sequence is also crucial to achieve high rates of art-sRNAs-based antiviral protection. Studies have shown that targeting highly conserved regions of viral critical genes is more successful than targeting various virus genes for antiviral silencing induction in plants. To elaborate further, critical multifunctional genes like replication-associated proteins, silencing suppressors and movement proteins are key players in the virus life cycle (Fondong 2013; Cisneros and Carbonell 2020; Bahari *et al.* 2022; Akbarimotlagh *et al.* 2023; Bahari *et al.* 2023). Targeting these genes may ensure robust resistance. Using these genes may lead to a more prolonged silencing effect as they possess highly conserved functional motifs so that viruses avoid the effect of selection pressure. This also helps produce escaped virus variants with silent mutations. Additionally, targeting multiple conserved motifs within one or more key viral proteins can greatly enhance the efficiency of this strategy (Carbonel *et al.* 2019a, b; Cisneros and Carbonell 2020).

Clustered regularly interspaced short palindromic repeats (CRISPR)

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas) together create a unique adaptive immune system present in archaea and bacteria. This system protects against foreign nucleic acid elements, like phages and plasmids, that are acquired horizontally by incorporating pieces of the invader's genetic material into their own genome, which allows them to thwart future

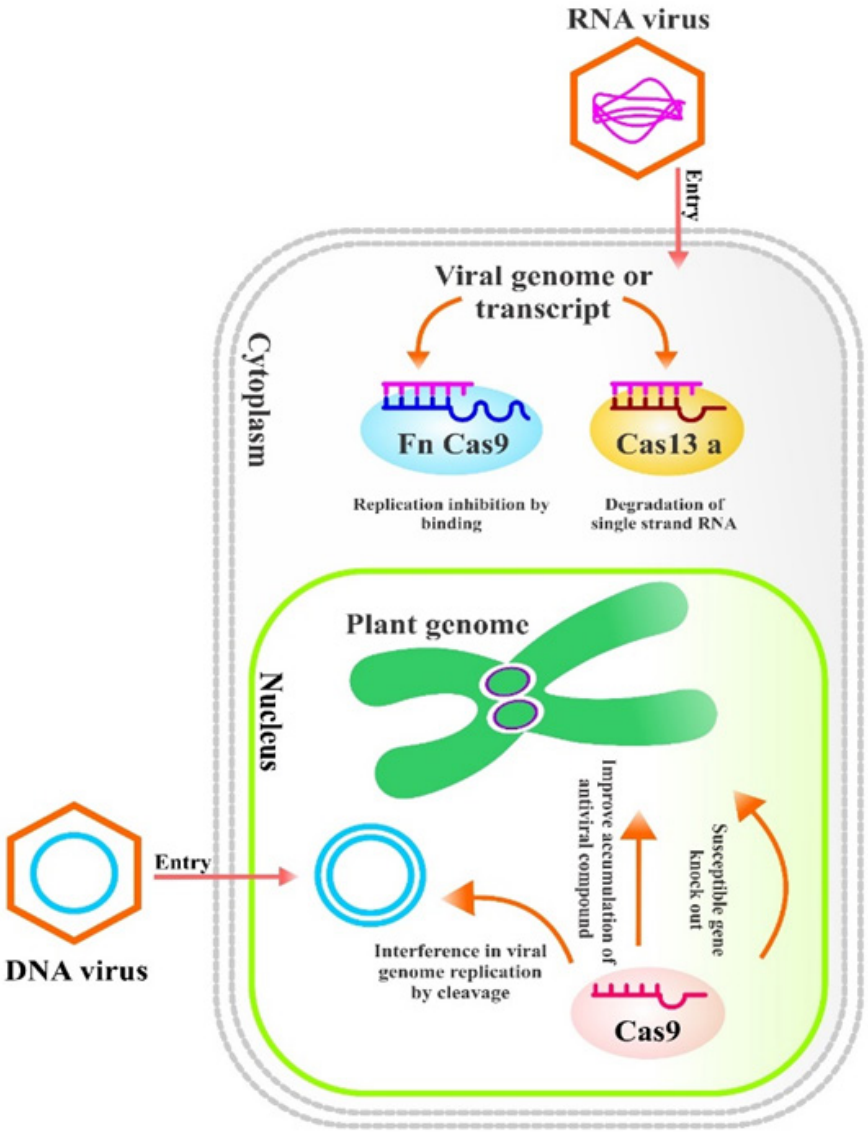

Fig. 3. Schematic illustration of CRISPR/Cas-based approaches used to engineer plants for virus resistance. The CRISPR/Cas system uses guide RNA (gRNA) and its associated protein (Cas) to interfere with plant viruses, either directly or indirectly. Direct interference can occur in DNA viruses during the amplification step through cleaving mediated by gRNA/Cas9, or in viral transcripts and RNA viruses by cleaving or binding, respectively, by gRNA/FnCas9 and gRNA/Cas13a. Indirect plant virus genome editing-mediated resistance is achieved by manipulating the plant genome. This can be done by inducing the production of antiviral compounds or introducing mutations in critical host factors required for completing the viral disease cycle

invasions (Fig. 3) (Bhaya *et al.* 2011; Terns and Terns 2011; Wiedenheft *et al.* 2012; Jinek *et al.* 2013; Chandrasekaran *et al.* 2016; Zaidi *et al.* 2016; Zhang *et al.* 2018; Cao *et al.* 2020; Jeyaraj *et al.* 2023). In 2012, it was demonstrated that CRISPR, associated with Cas9 nuclease from *Escherichia coli* (*E. coli*), could revolutionize genome editing research (Jinek *et al.* 2013). To perform genome editing using this system, scientists employ a guide RNA (gRNA) and a nonspecific endonuclease, typically Cas9. The short sgRNA (sgRNA) directs the Cas enzyme to scan the target genome, locate the substrate sequence, and cleave it. The cell's repair machinery then finalizes the process by repairing the broken double-strand DNA (Bhaya *et al.* 2011; Terns and Terns 2011 Cao *et al.* 2020). To provide more information, the DNA-cutting activity of this system involves a 20-22 nucleotide sequence that is complementary to the 5' end of the guide RNA, followed by a Protospacer Adjacent Motif (PAM) sequence to make recognition and cleavage by *Cas* endonuclease possible (Jeyaraj *et al.* 2023; Terns and Terns, 2011). The CRISPR/Cas9 system has become a popular choice for developing virus-resistant strategies (Bhaya *et al.* 2011; Sandhya *et al.* 2020; Zaidi *et al.* 2016; Jeyaraj *et al.* 2023) for the following reasons: (1) in contrast to other genome editing technologies like zinc finger

nucleases and TALEN (Transcription Activator-Like Effector Nucleases), the CRISPR/Cas9 system allows for reprogramming with minimal alterations to the guide RNA (gRNA) (Jinek *et al.* 2013) and eliminates the necessity for complex protein engineering laboratories (Zaidi *et al.* 2016; Zhang *et al.* 2018), (2) it carries less environmental risks due to its capacity of more minimal manipulation in the host (e.g., plant) genome than other tools, (3) its potential for non-transgenic delivery, which is discussed further in the non-transgenic method section, and (4) it has appeared to generate desired changes with greater precision than other crop improvement tools (Jeyaraj *et al.* 2023; Zaidi *et al.* 2016).

Applications of CRISPR

From the viewpoint of genome editing, resistance to viral pathogen could be obtained through two strategies. First, plant genome engineering and the second, viral gene knock-out (Chandrasekaran *et al.* 2016; Zaidi *et al.* 2016; Zhang *et al.* 2018; Kuroiwa *et al.* 2022; Jeyaraj *et al.* 2023). The first strategy is applied either by introducing a mutation in the host factors which are in charge of susceptibility against viral pathogens to help complete their host-dependent life cycle leading to the distribution of virus-host interaction or by manipulating the plant genome to produce the antiviral compound (Chandrasekaran *et al.* 2016; Sharma and Vakhlu 2021; Kuroiwa *et al.* 2022; Jeyaraj *et al.* 2023). This approach has shown great promise in developing germplasms that can resist biotic stress. Besides provoking the plant defense system against biotic stress, this resistance phenotype is recessive which could guarantee a more durable resistance than that derived from the dominant genes and are exposed to natural selecting pressure. Alternatively, the plant genome can be manipulated to produce antiviral compounds (Chandrasekaran *et al.* 2016; Kuroiwa *et al.* 2022). In their 2016 study, Chandrasekaran *et al.* demonstrated a successful application of CRISPR/Cas9 technology in producing resistant crops. By mutating eukaryotic initiation factor 4E (eIF4E) in cucumber plants they were able to confer resistance against a range of broad RNA viruses including *Cucumber vein yellowing virus* (CVYV) from the family *Ipomoviridae*, *Zucchini yellow mosaic virus* (ZYMV) and *Papaya ring spot mosaic virus-W* (PRSMV-W) from the genus *Potyvirus*. Notably, the edited part of the genome was inherited by the resultant progenies (Chandrasekaran *et al.* 2016). In line with the recent study, to combat *Pepper venial mottle virus* (PVMV), an isoform of eIF4E has been inactivated in tomato cultivars using a CRISPR/Cas9 construct (Kuroiwa *et al.* 2022). Observation of the resistance phenotype was noted in potato plants infected with *Potato virus Y* (PVY) following the expression of CRISPR/Cas9 from an *eIF4E* targeting cassette (Noureen *et al.* 2022). A comparable study was conducted to knock out the eIF4E isoform to control melon's *Moroccan watermelon mosaic virus* (MWMV) (Pechar *et al.* 2022). In accordance with this recent research, a CRISPR/Cas9 construct was used to deactivate another isoform of *eIF4E* in tomato cultivars to combat the *Pepper veinal mottle virus* (PVMV) (Moury *et al.* 2020). According to Gomez *et al.* (2019), a mutation in nCBPs proteins, specifically cassava *eIF4E* isoforms, induced by CRISPR/Cas9 technology can improve the protection of cassava plants against dual infection by *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) (Gomez *et al.* 2019). As mentioned earlier, another beneficial aspect of plant genome engineering is provoking plants to express more antimicrobial metabolites. In line with this, a CRISPR/Cas9 construct simultaneously targeting isoflavone genes, when expressed in soybean plants enhanced flavonoid accumulation and consequently conferred resistance to soybean mosaic virus (SMV) (Zhang *et al.* 2020).

Most studies on the genome engineering of DNA viruses using CRISPR /Cas technology have focused on members of the *Geminiviridae* family. However, other DNA viruses have also been targeted and promising results have been obtained from most experiments on both model and crop plants. For instance, when an engineered CRISPR/Cas9 specific for TYLCV was used to knock out the CP gene, tomato plants showed a high degree of resistance (Tashkandi *et al.* 2018). An effective antiviral resistance was established by Kis and co-workers who expressed, in barley lines, four manipulated CRISPR/Cas9 constructs containing WDV conserved segments (encoding Rep, CP, MP and long intergenic region (LIR) region) targeting the virus subgenomic (sg) RNA as well as exclusively functioning in monocotyledons. It was realized that lines harboring the constructs gave a robust resistance against this destructive virus which was also maintained in the progenies (Kis *et al.* 2019). By contrast, a study conducted on transgenic cassava plants expressing CRISPR/Cas9 machinery failed to achieve resistance due to the emergence of new *African cassava mosaic virus* variants that were not susceptible to the cleaving activity. This has led to suggestions to design the CRISPR/Cas9 system more precisely to prevent aiding the viral evolution to become more aggressive (Mehta *et al.* 2019). Another study on the *pararetrovirus, Banana bunchy top virus* which is integrated into the host plant genome showed that introducing a mutation in *Musa* spp., transformed for the expression of CRISPR/Cas9, resulted in successful protection (Tripathi *et al.* 2019).

In addition to DNA viruses (Tashkandi *et al.* 2018; Kis *et al.* 2019; Tripathi *et al.* 2019) applying a CRISPR/Cas9 approach for editing viral gene is well-developed

for RNA viruses. Taking advantage of special trait features of Cas13a nuclease from *Leptotrichia wadei* (*L. waidei*) (LwaCas13a) and *Leptotrichia shahii* (*L. Shahii*) (LshCas13a), along with Cas13d from *Ruminococcus flavefaciens* (*R. flavefaciens)* endonuclease, researchers have been able to remove the constraints of interfering with RNA viruses (Fig. 3). This breakthrough has opened up new possibilities for controlling RNA viruses by allowing for the modification of the single-strand RNA sequences. This interference can either result from insertions or deletions in either viral RNA or the transcript (Aman *et al.* 2018a, b; Konermann *et al.* 2018; Zhang *et al.* 2019). Furthermore, FnCas9 derived from *Francisella novicida (F. novicida*) prevents RNA virus activity by binding to it, rather than by cleaving (Zhang *et al.* 2018, 2019). When cucumber crops were transferred with reprogrammed FnCas9 protein and its associated gRNA construct, the accumulation and symptoms of TMV and CMV were significantly attenuated (Zhang *et al.* 2018). The CRISPR/Cas13a system has been expressed from rice plants to edit the genome of *Southern rice black-streaked dwarf virus* (SRBSDV) dsRNA. This was done using a monocotyledon promoter which resulted in the inhibition of viral replication and low SRBSDV accumulations. They also achieved a similar result using the FnCas9-based cassette instead (Zhang *et al.* 2019). Utilizing the CRISPR/Cas13a cassette carrying capacity of multiple sequence targeting conserved regions in some strains of PVY has produced efficient and broadly resistant *Solanum tuberosum* (*S. tubersum*) against PVY strains (Zhan *et al.* 2019; Noureen *et al.* 2022).

Non-transgenic resistance eliciting methods

Despite remarkable advancement and significant improvements in developing transgenic plants harboring virus resistance traits, a limited number of them are reaching the agricultural markets (Table 1). Accordingly, the major constraint for the commercial production of GMO crops is the strict environmental safety regulations which make the commercialization process very time-consuming (Ahmad *et al.* 2023). Also, the fact that not all crop plants are transformable is another reason for this limitation (Gaffar and Koch 2019). Plant protection scientists are exploring alternative methods for triggering resistance in plants, without the use of genetic modification (Das and Sherif 2020; Rêgo-Machado *et al.* 2023). A popular approach is known as dsRNA topical application, which involves applying dsRNA, hpRNA, or siRNA directly to the surface of the plant (Table 2). This can be done through such methods as spraying, infiltration, mechanical inoculation, injection, dropping, and spreading. The plant then absorbs the dsRNA and triggers RNA silencing pathways. To ensure effectiveness, several factors need to be considered, such as the type of virus being targeted, the size and dose of the triggering dsRNA sequence, the genomic region of the target sequence, the appropriate application method and timing, the delivery technique and the suitable plant organ for application, as well as the formulation of the trigger dsRNA sequence (Gaffar and Koch 2019; Das and Sherif 2020).

Although there is still a lack of global consensus on whether CRISPR-edited crops are considered non-GMO products, there is an alternative method to address the presence of non-related DNA sequences in genetically modified plants. This method involves using a CRISPR genome editing system that depends on the type of recurring Double-Strand Break (DSB) repairing system, with the possibility of making minimal genome modifications limited only to changing desired nucleotides through deletion or insertion. The Site-Directed Nuclease (SDN) 1 and SDN 2 repair the CRISPR system-mediated broken double-stranded DNA based on non-homologous end joining and homologous-directed repair. This means that the edited genome cannot be distinguished from the wild-type genome. In some countries, these two classes of CRISPR systems are widely accepted and do not need to go through the regulation process (Wolt *et al.* 2016; Hjort *et al.* 2021; Ahmad *et al.* 2023).

The newly emerging Ribonucleoprotein (RNP) technique is widely being proposed to directly deliver the CRISPR system-associated biomolecules, Cas9 nuclease protein and guide RNA, to the host target cell which is named as next generation CRISPR. Delivery of CRISPR/Cas9 RNP molecules through biolistic and polyethylene glycol (PEG)-mediated transformation has been optimized for plant cells. However, an ideal RNP packaging material must possess optimum properties, including a protective effect from protein and nucleic acid degradation, and it must easily be embedded into the cells. Conjugated negatively charged magnetic nanoparticles (MNP) for covering CRISPR/Cas9 RNP harboring a positive charge is a potential alternative for direct transfer into plant pollen, omitting the conventional plant transformation (Zhang *et al.* 2021).

To achieve a guaranteed direct delivery of resistance-eliciting biomolecules (such as miRNA, RNAi, and CRISPR/Cas), two factors need to be considered. First, the biomolecules need to be packaged into a proper carrier; and second, an optimized delivery method needs to be employed. There is a growing interest in developing transgene-free engineered plant pathogen-resistant germplasms. Innovative, efficient materials and methods are being developed to direct the delivery of biomolecules into plant cells

(Zhang *et al.* 2021; Ahmad *et al.* 2023). Currently, nano-carrier-based materials are receiving special attention due to their safety, cost-effectiveness, and simplicity in delivering resistance-eliciting biomolecules into plant cells (Hogenhout *et al.* 2009; Mitter *et al.* 2017; Mout *et al.* 2017; Mujtaba *et al.* 2021). The carriers for transferring genetic material to the plant can be introduced through physical approaches such as biolistic particle bombardment, microinjection, and electroporation to the protoplast, or chemical approaches such as polyethylene glycol (PEG)-mediated protoplast transformation and pollen magnetofection-mediated delivery (Zhang *et al.* 2021; Jeyaraj *et al.* 2023). Yet, another possible method is the viral RNA vector-mediated delivery, which has a potential for mechanical inoculation (Ariga *et al.* 2020; Deb *et al.* 2022).

Conclusions

PDR was developed on the basis of a hypothesis of cross protection in the mid-1980s and since then there has been an evolution of the biotechnological approaches to manage plant viral diseases. All of these approaches are accompanied by drawbacks although it seems that a newer method always overweighs the previous one. CRISPR, as the most recent procedure, also has a limit in the sense that the next generation can be applicable with vegetatively-propagated crops such as potatoes, onions, ornamentals, etc. But the good thing is that we now have several technologies compared to a single or only few approaches as were in the past. This offers flexibility in selecting one of these approaches by considering factors such as robustness of the method in a given place, the crop itself, the costs involved and the socio-economic conditions. The story is still going on and one of the future research areas would be to find genes other than *eIF4e* which contribute to virus replication or facilitate its infectivity in the host plant.

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